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(54) Title: DIAGNOSING NEUROLOGIC DISORDERS

(57) Abstract

    Methods are described for identifying protein profiles which are specific for neurologic disorders. Methods of diagnosing neurologic disorders by detecting the levels of individual proteins in the disease–specific protein profile are also provided. Disease–specific protein profiles for Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis and schizophrenia are described.

DIAGNOSING NEUROLOGIC DISORDERS

CROSS-REFERENCE TO RELATED APPLICATIONS

This invention claims priority to United States Provisional Patent Application Serial No. 60/036,586 filed March 14, 1997 and United States Provisional Patent Application Serial No. 60/061,402 filed September 30, 1997.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

This invention was made in part during work supported by a grant from the USPHS # NS30531. The government has certain rights in this invention.

TECHNICAL FIELD

This invention relates to diagnosis of neurologic disorders. More specifically, the invention provides methods of obtaining a disease-specific protein profile by determining proteins from a biological sample which are altered relative to normal controls. The invention also relates to methods of diagnosing neurologic disorders by determining the levels of the proteins which make up the disease-specific protein profile. The invention provides methods of diagnosing schizophrenia, Alzheimer's disease, Parkinson's disease, multiple sclerosis, subacute sclerosing panencephalitis (SSPE) and other neurologic disorders using disease-specific protein profiles.

BACKGROUND

Neurologic disorders such as schizophrenia, manic depression (bipolar disorder), Alzheimer's Disease, Parkinson's disease, multiple sclerosis, Huntington's disease and bacterial meningitis, are major public health concerns. Alzheimer's Disease (AD), for example, affects over 4 million Americans while schizophrenia affects approximately 2 million Americans. Alzheimer's disease has a gradual onset, usually beginning later in life. Symptoms of AD include difficulty with memory and loss of intellectual abilities severe enough to interfere with routine work or social activities. Confusion, language problems (such as trouble finding words), poor or decreased judgment, disorientation in place and time and changes in behavior or personality also occur. Eventually, AD leaves its victims

totally unable to care for themselves. Currently, the only way to confirm a diagnosis of AD⁻ is by post-mortem autopsy.

       Parkinson's Disease (PD) affects over 1 million people in the U.S., with 20 new cases of PD per 100,000 people per year. PD is a chronic disease in which certain
5   dopamine-producing neurons are destroyed. There is no definitive pre-mortem diagnosis available for PD. A diagnosis is based on a neurological examination, the severity of the symptoms and brain scans (CT or MRI) to help rule out other neurological diseases. If the symptoms are significant, a trial test of anitparkinsonian drugs may be used to diagnose the disease. Primary symptoms include stiffness, tremor, slowness and poverty of movement,
10   difficulty with balance and walking. Secondary symptoms are depression; sleep disturbances; weight loss; dementia; drooling; difficulty with speech, breathing or swallowing; constipation and stopped posture.

       Schizophrenia usually develops between adolescence and age 30 and is characterized by positive symptoms (delusions or hallucinations), negative symptoms
15   (blunted emotions and lack of interest), and disorganized symptoms (confused thinking and speech, or disorganized behavior and perception). Schizophrenia can generally be treated with antipsychotic drugs which control most of the clinical symptoms of the disease.

       Early and accurate diagnosis of neurologic disorders is essential for preventing
20   relapses, selecting and monitoring the appropriate treatment regime, and reducing the risk of substance abuse and suicide. However, there are currently few specific and accurate molecular markers which would allow for diagnosis of AD, PD or schizophrenia in a living subject. Currently, neurologic disorders are usually only tentatively diagnosed by pre-mortem clinical evaluation or, definitively, by post-mortem autopsy of the brain.
25   However, clinical evaluation of neurologic disorders is complex as the physician must rule out other problems or disorders which exhibit like symptoms. For example, PD can be confused with multiple sclerosis, ALS or Huntington's chorea. In the case of schizophrenia, drug abuse, seizures, major depressive or manic episodes, autism, and other personality disorders may present with psychotic symptoms similar to those seen in
30   schizophrenia. Brain tumors, stroke, Alzheimer's and Parkinson's diseases can all present with symptoms including loss of memory, personality changes and aphasia.

The search for protein markers which are specific for a neurological disorder has
proven elusive. Using two-dimensional gel electrophoresis (2DE), Hsich *et al.* (1996)
NEJM 335(13):924-929 report that transmissible spongiform encephalopathies can be
detected by the presence or amount of the protein in cerebrospinal fluid. U.S. Patent No.

5   4,874,694 to Gandy et al. claims a method of diagnosing certain neurologic disorders by
incubating cerebrospinal fluid from a subject with 32-P labeled adenosine triphosphate
(ATP) and a protein kinase capable of transferring phosphate from the ATP. Gel
electrophoresis is then performed on the proteins and the autoradiography obtained
compared to autoradiographs of known pathologies.

10   There are also references that describe diagnosis of neurologic disorders using only
one biochemical marker. Most references describe one specific marker which is used for
multiple neurologic disorders and where the specificity is low. The present invention has
high specificity, for instance greater than 98% in CJD.

U.S. Patent No. 5,006,462 to Gattaz et al. describes diagnosis of schizophrenia by

15   measuring increased enzymatic activity of phospholipase A2 (PLA2) in plasma samples
when compared to nonschizophrenic controls. However, this assay could differentiate only
approximately 70% of schizophrenic patients from healthy controls.

WO 95/05604 discloses methods of diagnosing Alzheimer's disease by detecting
the presence of proteins altered in the olfactory neuroblast lysates of AD patients. WO

20   95/05395 discloses a method of diagnosing AD by measuring elevated levels of alpha-1
anti-trypsin or fibrinogen. U.S. Patent No. 5,429,947 to Merril et al. describes a method
for diagnosing schizophrenia and Alzheimer's Diseases (AD) by detecting elevated levels
of specific haptoglobin proteins in the bodily fluids of patients. This method is not specific
for either of the two disorders listed. U.S. Patent No. 5,234,814 describes a method for

25   assisting the diagnosis of AD by detecting amyloid precursor related proteins (APP) using
an antibody which is reactive with the C-terminal of human amyloid precursor protein.
Japanese Patent Nos. 3211461 and 2543606 disclose diagnosis of multiple sclerosis by
measuring the amount of human tumor necrosis factor-alpha (h-TNF) in cerebrospinal
fluid.

30   In diseases of the nervous system, the blood-brain barrier prevents diffusion of
proteins from the brain to the peripheral blood system. However, the cerebrospinal fluid
(CSF) is in immediate contact with the nervous system and proteins from this fluid can be

used as the molecular measure of pathology and responses to therapy. High resolution, two dimensional (2DE) separation techniques have yielded a detailed protein map of more than 2,000 individual components from CSF samples. The first dimension of 2DE separates proteins on the basis of charge, while the second dimension separates based on mass.

The present invention provides a specific method of generating disease-specific molecular markers. The disease-specific markers are obtained by screening a large number of proteins from a biological sample, identifying the particular proteins which are altered in subjects with neurological diseases by comparing their levels to levels found in normal subjects. Individually, these proteins do not distinguish the disorders from normal or other disease controls, but in combination, these protein profiles are very accurate at differentiating specific disorders. The particular proteins can then be isolated, purified, and used to generate antibodies. The antibodies are then used, for example, in Western blots or other immunoassays to diagnosis a specific disease condition in a sample. The disease-specific protein profiles are also called "bar-codes" in reference to the unique marking system used for merchandise. As with merchandise, the profiles expressed as bar codes provide a unique marking system for neurologic disorders. The bar-coding strategy of this invention allow for easy, rapid and specific assay for neurologic disease including schizophrenia, manic depressive disorder, Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS) and Creutzfeldt-Jakob disease (CJD). It was the surprising discovery of the inventor that when the levels of the protein profile obtained from a patient is compared to normal controls, neurologic disorders can be diagnosed.

SUMMARY OF THE INVENTION

The present invention provides a method of diagnosing a neurological disorder in a biological sample, the method comprising: (a) obtaining a disease-specific protein profile by detecting proteins whose levels are altered in a subject having a neurological disorder when compared to levels of a healthy control; (b) obtaining the biological sample; (c) measuring the levels of the disease-specific protein profile obtained in step (a) in the biological sample; and (d) comparing the levels of the disease-specific protein profile in the biological sample to levels found in the healthy control; wherein altered levels of the

protein profile of the biological sample as compared to the healthy control is diagnostic of the neurological disorder.

In a preferred embodiment, the biological sample is cerebrospinal fluid. In another preferred embodiment, the neurologic disorder is selected from the group consisting of Alzheimer's Disease (AD), schizophrenia, Parkinson's disease (PD), multiple sclerosis, subacute sclerosing panencephalitis (SSPE) and transmissible spongiform encephalopathies. In a particularly preferred embodiment, the neurologic disorder is AD and the protein profile comprises proteins designated AD-1 through AD-18. In another particularly preferred embodiment, the neurologic disorder is PD and the protein profile comprises CIT-A, CIT-B, CIT-C (protein 129), CIT-1 (alpha 1 microglobulin subunit), CIT-2 (PGDS isoform), CIT-3 (PGDS isoform), CIT-4 (complement 4 gamma - C4γ) and CIT-5 (Apo-A1 lipoprotein). In yet another particularly preferred embodiment, the neurologic disorder is schizophrenia and the protein profile comprises protein 91, protein 48, protein 46, orosomucoid, prostaglandin D synthetase (PGDS), antithrombin III and fibrinogen degradation products. In other particularly preferred embodiments, the neurological disorders are multiple sclerosis, subacute sclerosing panencephalitis (SSPE), or Creutzfeldt-Jakob disease (CJD).

In another preferred embodiment, the disease-specific protein profile is obtained using 2 dimensional gel electrophoresis (2DE), Western blotting or immunoassay. In another preferred embodiment, the protein profile is measured by visualizing a 2DE gel, Western blotting or by immunoassay.

As will become apparent, preferred features and characteristics of one aspect of the invention are applicable to any other aspect of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a color reproduction of a 2DE gel electrophoresis of CSF fluid obtained from normal controls. Five positional standards, or landmarks, are identified in green. The position of five spots which are qualitatively or quantitatively altered in CSF from patients with disease are shown in red and labeled CIT-1 through CIT-5 in blue.

Figure 2 is a color reproduction of a 2DE gel electrophoresis of CSF fluid obtained from Parkinson's disease patients. Each of the protein spots comprising a PD-specific protein profile are marked in red.

Figure 3 is a color reproduction of a 2DE gel electrophoresis of CSF fluid obtained-
from Alzheimer's Disease patients. Eighteen spots comprising a AD-specific protein
profile are marked in red.

Figure 4, panels A through C, are plots depicting the relative quantity of CIT-5

5   (Apo-A1 lipoprotein) in various Parkinson's patients. Each bar is representative of one
patient. Panel A shows CIT-5 (Apo-A1 lipoprotein) plotted against the "H + Y" rating, a
standardized index of disease severity. As disease severity increases, the amount of CIT-5
(Apo-A1 lipoprotein) decreases. On the x-axis, a "3" denotes long duration PD with
dyskinesia, and moderate to high dopaminergic therapy, a "2" denotes medium duration,

10   dyskinesia and low dopaminergic therapy and a "1" denotes short duration disease, no
dyskinesia and no or minimum dopaminergic therapy. Panel B shows CIT-5 (Apo-A1
lipoprotein) plotted against the age of patient/duration of clinical disease. On the x-axis,
"OL" refers to patients in whom the clinical disease onset occurred when they were older
(above 50) with a long duration of disease, "YL" refers to a young-onset patients (below

15   50) with a long duration of disease, "OS" refers to old-onset and short duration disease,
"YS" refers to young-onset and short duration. Panel C shows CIT-5 (Apo-A1
lipoprotein) plotted versus clinical parameters. The numbers on the x-axis are the same
classifications as for Panel A. All panels in Figure 4 show that as PD severity or duration
increases, the amount of CIT-5 (Apo-A1 lipoprotein) decreases.

20   Figure 5 is a graphical representation of a multiple discriminant function analysis
plotting canonical discriminant functions obtained from the protein profiles of six PD
patients (c); six AD patients (a) and two controls (b).

Figure 6 is a graphical representation of a multiple discriminant function analysis
plotting canonical functions obtained from the protein profiles of six PD patients (c); four

25   AD patients (b) and six controls (a).

Figure 7 shows the effect of various schizophrenia drug therapies on the level of
protein 91. In normal controls, protein 91 has a normalized protein quantity (density units)
of approximately 8. Individuals effected with schizophrenia have only around 4 units of
the same protein. Patients on any of the three therapy regimes tested have less protein 91

30   than untreated or normal individuals.

Figure 8 shows the effect of various schizophrenia drug therapies on the level of
protein 48. In normal controls, protein 48 has a normalized protein quantity of

approximately 7. Individuals affected with schizophrenia have around 13 units of the same

protein. Patients on any of the three therapy regimes tested show a protein 48 level which

is less than untreated individuals, but closer to the normal levels.

Figure 9 shows the effect of various schizophrenia drug therapies on the level of

one isoform of prostaglandin D synthetase (PGDS). In normal controls, this PGDS

isoform has a normalized protein quantity of approximately 8. Patients affected with

schizophrenia have less than 4 units of the same protein. Samples obtained from patients

on any one of the three therapy regimes show that PGDS levels remain lower than normal

levels.

Figure 10 shows the effect of various schizophrenia drug therapies on the level of

protein 46. In normal controls, protein 46 has a normalized protein quantity of

approximately 8. Individuals affected with schizophrenia have around 13 units of the same

protein. Patients on any of the three therapy regimes tested show a protein 46 level which

were intermediate between untreated and normal levels.

Figure 11 depicts a two dimensional graph in which samples have been positioned

based on their protein profile. A diagnosis of SSPE was made in sample labeled "E". This

graph shows complete resolution of samples diagnosed as characteristic of SSPE from

other samples, including multiple sclerosis patients.

MODES FOR CARRYING OUT THE INVENTION

Throughout this application, various publications, patents, and published patent

applications are referred to by an identifying citation. The disclosure of the publications,

patents, and published patent specifications referenced in this application are hereby

incorporated by reference into the present disclosure to more fully describe the state of the

art to which this invention pertains.

As used herein, certain terms will have specific meanings.

The term "neurologic disorder" is intended to refer the diverse group of disorders

which affect the central or peripheral nervous system. The disorders may be acute or

chronic and include, but are not limited to, schizophrenia, manic depression, Alzheimer's

disease, Parkinson's disease, multiple sclerosis, transmissible spongiform encephalopathies

(Creutzfeldt-Jakob disease in humans, mad cow disease in bovines and scrapie in sheep),

stroke, herpes simplex encephalitis, subacute sclerosing panencephalitis (SSPE), and brain-damage associated with cerebral infarction.

As used herein, the terms "Alzheimer's Disease" and "AD" are used interchangeably to include the mental disorders characterized by memory loss, disorientation, confusion and the like. AD is also characterized by amyloid plaques and neurofibrillary tangles in the brain, which can be seen upon autopsy.

The terms "Parkinson's Disease" or "PD" are used interchangeably to include the neurologic disease characterized by tremor, rigidity, akinesia (freezing), postural disturbance or balance problems. Fatigue, depression, anxiety, restlessness, poor handwriting, quieting of voice, change in posture, muscle aches, cramping or paresthesias (tingling feelings) are also reported. Diagnosis of PD can be complicated because of "Parkinsonism look-alikes" or conditions which share clinical features with PD. PD can be divided into different subtypes based, for example, on age of onset, duration or amount of dyskinesia.

The term "schizophrenia" is intended to include the group of mental disorders characterized by disruptions in thinking and perception. In a clinical evaluation, schizophrenia is commonly marked by auditory hallucinations (especially hearing voices), disorganized thought processes, delusions, and the absence of emotion or affect. Clinical diagnosis of schizophrenia is complex, and as with most psychiatric diagnostic categories, is effected at the syndromal level. (see, Michael Flaum, (1995) "The diagnosis of schizophrenia" in CONTEMPORARY ISSUES IN THE TREATMENT OF SCHIZOPHRENIA, Shriqui and Nasrallah eds., American Psychiatric Press, Inc, Washington D.C.).

The disorder known as schizophrenia can be divided into several subtypes or categories. The term "schizoaffective" is intended to mean a category of schizophrenia patients who also exhibit mood disorders, for example, bipolar disorder. Schizoaffective disorder is generally characterized by common schizophrenia symptoms in combination with major depressive or manic episodes. "Post partum" schizophrenia refers to a the category of schizophrenia which exhibits common symptoms but onsets in women after giving birth. The term "schizophrenic" refers to a person who suffers from schizophrenia.

The term "transmissible spongiform encephalopathies (TSEs)" is intended to refer to a group of neurodegenerative diseases. In humans, these diseases include Creutzfeldt-Jakob disease (CJD), Gerstmann-Staussler-Scheinker syndrome, Fatal Familial Insomnia,

and Kuru. *(see, e.g.,* Brown *et al.* in NEURODEGENERATIVE DISEASES, Calne *ed.,* W.B.
Saunders, Philadelphia (1994); Medori *et al.* (1992) *N. Engl. J. Med.* 326:444-449). In
animals, the TSEs include sheep scrapie, bovine spongiform encephalopathy, transmissible
mink encephalopathy, and chronic wasting disease of captive mule deer and elk (Gajdusek

5   (1990) *Subacute Spongiform Encephalopathies: Transmissible Cerebral Amyloidoses
Caused by Unconventional Viruses.* pp. 2289-2324 In: *Virology,* Fields, ed. New York:
Raven Press, Ltd.). All TSEs are characterized by the same hallmarks: a spongiform
degeneration, reactive gliosis in the cortical and subcortical gray matters of the brain, and
transmission when experimentally inoculated into laboratory animals including primates,

10   rodents and transgenic mice.

        A biological "sample" as used herein includes a variety of sample types obtained
from an individual and is typically used in a diagnostic procedure or assay. The definition
encompasses blood, cerebrospinal fluid (CSF) and other liquid samples of biological
origin, solid tissue samples such as biopsy specimen or tissue cultures or cells derived

15   therefrom and the progeny thereof. Methods of collecting and storing tissue or fluid
samples are known to those of skill in the art. The definition also includes samples that
have been manipulated in any way after their procurement, such as by treatment with
reagents, solubilization or enrichment for certain components, such as proteins or
polynucleotides. The term "biological sample" encompasses a clinical sample, and also

20   includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid and
tissue samples.

        The terms "level" or "levels" are used to refer to the presence and/or amount of
protein, and can be determined qualitatively or quantitatively. A "qualitative" change in
the protein level refers to the appearance or disappearance of a protein spot that is not

25   detectable or is present in samples obtained from normal controls. A "quantitative" change
in the levels one or more proteins of the profile refers to a measurable increase or decrease
in the protein levels when compared to a healthy control.

        The term "protein profile" refers to a pattern of specific proteins whose levels are
altered in samples obtained from subjects with neurologic disorders when compared to a

30   healthy control. The profile may be expressed in many forms, including, for example, as a
"molecular bar code" composed of the proteins of the profile. A disease-specific protein
profile is obtained by comparing the level of a variety of proteins in a sample taken from a

healthy control to the levels found in samples taken from affected individuals. Individually, the proteins which are altered in the affected individuals may not be diagnostic of the disorder, but, when viewed together as a profile, are disease-specific. The proteins which comprise the profile may all be increased, decreased, present or absent with

5   respect to healthy levels. More often, some of the proteins will be increased and some will be decreased as compared to the control.

The proteins which make up the disease-specific protein profile may be identified by their common name, their structure, their sequence, by the antibodies which recognize them or by their migration pattern in gel electrophoreses. One or two dimensional gels

10   may be used. Methods of 2DE are known in the art, and described herein in Example 1. For example, a protein profile for AD comprising 18 proteins has been identified. Two of the proteins have been identified by their common names, Haptoglobin 1 precursor subunits, while sixteen are identified by their migration pattern in gel electrophoresis. A protein profile for PD comprises 9 proteins, including CIT-A, CIT-B, protein 129, alpha 1

15   microglobulin subunits, two PGDS isoforms, complement 4 gamma and Apo-A1 lipoprotein. The profile for schizophrenia described in the present application includes proteins 48, 46 and 91. To date, the amino acid sequence of these proteins is not known. Accordingly, these proteins are identified herein by randomly-assigned numbers corresponding to the location on a 2DE gel where the protein appears under the conditions

20   described herein. Proteins 5, 48, 46 and 91 have been previously identified in Harrington et al. (1985) *Clinical Chemistry* 31(5):724. As shown in Figure 1 of this reference, these proteins migrate in response to electrophoresis in a characteristic manner.

The proteins of the protein profile may also be identified, for example, by their isoelectric focusing point (pI) and molecular weight (MW) in kilodaltons (kD). For

25   example, some of the proteins identified herein have the following pI and apparent MW:

Table 1

Protein Number (Name)	pI	MW (kD)
protein 48	6.2	46
protein 46	6.0	48
protein 91	5.7	80
108	5.3	30
110	5.1	32
protein 2 (PGDS)	5.5	20
protein 5 (PGDS)	5.7	20
protein 129	6.1	25
CIT-A	5.2	94
CIT-B	5.58	54
CIT-C	5.54	26
CIT-1 (alpha 1 microglobulin subunit)	5.12	30.851
CIT-2 (PGDS isoform)	5.2	25
CIT-3 (PGDS isoform)	5.2	25
CIT-4 (complement 4 gamma)	6.37	33.074
CIT-5 (Apo-A1 lipoprotein)	5.46	23.320
AD-1	6.37	150
AD-2	5.46	65
AD-3	6	63
AD-4	6.09	63
AD-5	6.09	63
AD-6	64	6.6
AD-7	64	6.6
AD-8	62	6.9
AD-9	62	7
AD-10	51	5.46
AD-11 (Haptoglobin 1 precursor subunit)	42	5.47
AD-12 (Haptoglobin 1 precursor subunit)	42	5.47
AD-13	30.5	5.48
AD-14	30.5	5.48
AD-15	30	5.1
AD-16	30	5.1
AD-17	28	5.18
AD-18	17	6.68

The pI and molecular weight values are intended to include a range which includes any variations which may occur due to experimental conditions as would be known by one of skill in the art. In the neutral pH region, pIs will generally range up or down 0.5 from the value given. At higher pHs, the range may be $\pm$ 1. Molecular weight ranges will usually be within 5 kilodaltons of the value given, although glycoproteins may migrate

anomalously in SDS PAGE gels and give show MW values which are higher than the actual MW.

The proteins which comprise the disease-specific profiles can be quantified, for example, by measuring the units of protein concentration or normalized density units.

5   Alternatively, the protein profile can be visualized, for example, from electrophoresis, in particular, 2DE. Electrophoretic methods are well-known to those skilled in the art and are described in detail herein. Visualizing proteins may be performed by any method known in the art. Coomassie blue and silver stain, for instance, are well known methods of visualizing proteins on gels. Western blotting and autoradiography of

10   gels can also used to identify the disease-specific protein profiles.

It is intended that the protein profiles used in the methods described herein be composed of at least 2 different proteins, or alternatively, at least two isoforms or glycoforms of the same protein. Preferably the protein profiles are comprised of between 3 and 20 proteins or glycoforms, more preferably, between 4 and 15, and even more

15   preferably 5 to 10. Glycoforms of the same protein can be distinguished by methods known in the art. As described above, glycoforms of proteins are known to migrate differently in 2DE, as determined by molecular weight and isoelectric focusing points. Therefore, a protein profile could be prepared by measuring or visualizing glycoforms of the same protein. Although the only limit on the number of proteins which can make up a

20   profile is the number of proteins in the sample, for ease of analysis, less than 20 is preferred.

In addition to differentiating between neurologic disorders, it is also intended the protein profiles can be used to differentiate between diagnostic categories of individual diseases. In schizophrenia, for example, post-partum schizophrenia or schizoaffective

25   disorder may produce protein profiles which are distinct from conventional schizophrenia. Similarly, early-onset Alzheimer's disease may present with a different protein profile from the more common age-associated AD. Moreover, it is contemplated that an individual subject may have a unique protein profile, for instance, because they are suffering from more than one neurologic disorder. In addition, the protein profile can be

30   used to distinguish specific cell damage or brain region damage based on the appearance of proteins unique to their origin.

A "healthy control" or "normal control" is a biological sample taken from an individual who does not suffer from a neurologic disorder. A "negative control," is a sample that lacks any of the specific analyte the assay is designed to detect and thus provides a reference baseline for the assay.

As used herein, the term "cerebrospinal fluid" or "CSF" is intended to include whole cerebrospinal fluid or derivatives or fractions thereof well known to those of skill in the art. Thus, a cerebrospinal fluid sample can include various fractionated forms of cerebrospinal fluid or can include various diluents added to facilitate storage or processing in a particular assay. Such diluents are well known to those of skill in the art and include various buffers, preservatives and the like.

As used herein, the term "2-dimensional gel electrophoresis" or "2-D gel electrophoresis" or "2DE" is intended to refer to the two dimensional migration of proteins in solution or suspension in the presence of an electrical field. Methods of 2DE are well known to those of skill in the art. See generally, R. Scopes (1982) *Protein Purification,* Springer-Verlag, N.Y. and Deutscher (1990) *Methods in Enzymology Vol. 182: Guide to Protein Purification,* Academic Press Inc., N.Y.). For example, 2DE relies on isoelectric focusing (IEF) in either carrier ampholyte gradient gels or immobilized pH gradients for one dimension and SDS-polyacrylamide gels for the second dimension. The amount of polyacrylamide to be used in making the gels can be readily determined by a skilled artisan.

As used herein, an "immunoassay" is an assay that utilizes an antibody to specifically bind to the antigen. The immunoassay is thus characterized by detection of specific binding of the proteins of the protein profile to antibodies as opposed to the use of other physical or chemical properties to isolate, target, and quantify the analyte. Direct immunoassay and competitive immunoassays are described in detail below.

As used herein, an "antibody" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

The basic immunoglobulin (antibody) structural unit is known to comprise a tetramer or dimer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50–70 kD). The N–terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_l) and variable heavy chain (V_H) refer to these variable regions of the light and heavy chains respectively.

Antibodies may exist as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce $F(ab)'_2$, a dimer of Fab which itself is a light chain joined to V_H–C_H1 by a disulfide bond. The $F(ab)'_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the $F(ab)'_2$ dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (See, *Fundamental Immunology*, W.E. Paul, ed., Raven Press, N.Y. (1993) for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized *de novo* using recombinant DNA methodologies.

The phrase "specifically binds to a protein" or "specifically immunoreactive with," when referring to an antibody refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies preferentially bind to a particular protein and relative binding to other proteins does not occur in significant amounts. Specific binding to a protein under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, antibodies can be raised to the protein markers specific for schizophrenia. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid–phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. *See* Harlow and

Lane (1988) *Antibodies. A Laboratory Manual,* Cold Spring Harbor Publications, New
York, for a description of immunoassay formats and conditions that can be used to
determine specific immunoreactivity.

As used herein, the term "subject" refers to a mammal and includes, but is not

5   limited to, humans, bovine, mink, sheep, elk, cats and deer.

I. Obtaining Disease-Specific "Protein Profiles"

The present invention includes a method of obtaining a disease-specific protein
profile which may be expressed as a molecular bar code. The familiar bar code system of

10   grocery and other retail stores used to price goods reduces errors, saves time and helps
keep inventory. In the disease context, unique bar codes will provide accurate diagnosis,
patient management and therapeutic monitoring.

The protein profiles expressed as molecular bar codes are obtained from a
biological sample taken from a subject known to have a particular disorder, and preferably

15   a neurologic disorder. Sample collection is discussed in detail below. The proteins of the
sample are then detected. Preferably, the sample is first subjected to two-dimensional gel
electrophoresis (2DE) to distinguish individual proteins by their migration patterns. The
2DE gel is then visualized, preferably by silver staining and quantified, for example by
image analysis. Altered levels of particular proteins in the sample are then obtained by

20   comparing the protein levels in the sample to levels of a healthy control or a control known
to have a disorder, including a neurologic disorder. Preferably, a computer algorithm and a
database of protein levels from CSF is used to determine which particular proteins are
increased or decreased in the sample. In this way, from the thousands of proteins present
in biological samples, those which are altered in particular diseases can be identified. All

25   or some of the specific proteins which are altered are called the protein profile.

When 2DE is performed, it is preferable to use at least two different stained
electrophoresis gels to generate a protein profile. Protein spots which are present in every
gel are designated as positional standards or "landmarks." These landmark spots are
aligned as between at least two gels, and the rest of the spots digitized according to their

30   relation to the landmarks. Subsequently, a computer generated algorithm or the like can be
programmed to compare the test CSF with healthy controls and determine which spots are
different in one gel from another, and which are similar.

The algorithm used to determine the protein profile is a multi-step process. Computer programs can used to compare and contrast intensity of spots on 2DE gels, for instance commercially available programs such as "Melony" (BioRad). Development of computer generated algorithms is within the skill of an ordinary programmer. Generally, the protein spots on the 2DE gel are first identified using any spot-finding method known in the art. Preferably, an edge detector or peak identifier is used. Various parameters are recorded and stored for each spot, including, for example, x and y co-ordinates corresponding to pH and molecular weight along with total integrated density. Next, all spots are compared from one sample to one another, using a master gel retaining all data. The protein spots are either matched or unmatched and noted by their location on the gel. The differences can be qualitative (e.g. unmatched spots) or quantitative (statistically significant variations of normalized protein quantity).

The compared gel data is then split into groups of each disease and normal controls. Similarities and differences are noted as described above. All of the different proteins are then further analyzed in subsets of patients within each disease compared to controls.

The above-described steps each use conventional statistics for judging differences in data. Preferably, means, standard deviations, a student T-test, and analysis with appropriate degrees of freedom are employed. Other methods known to those in the art may also be suitable. Any changes are verified from one population by testing in a second population and only those changes that are replicable are confirmed as valid.

Using the novel method described here, we can define a diagnostically powerful profile for each disease and even for disease sub-types. This is achieved simply when an individual protein is so discriminative, but this is rare and likely to be unusual. One known example is the 14-3-3 marker for CJD. More commonly, a number of protein spots are altered in a statistically significant manner between disease and controls, but with overlap that is not useful for any one individual protein, but when analyzed together possess powerful discrimination. This can be achieved in a number of different ways, but most appropriately using multi-dimensional discriminant function analysis. Preferably, the algorithm is standard statistical software from Unistat. Other statistical methods will be known to those skilled in the art. Either linear or nonlinear discriminant function analysis can be performed, and alternative approaches can include principal component analysis, factor analysis, or cluster analysis. All variable proteins were examined for combinations

that would provide the best discrimination and then the best discrimination is investigated
for the least number of proteins. Once this is achieved (usually 10 or less proteins with
most discrimination coming from the first 5 proteins) then these proteins were used to test
a second, new population of samples. Only when these same proteins gave discriminating

5   power in the second population was this group of proteins considered for the profile. Once
so identified, the profile can be used to define a multidimensional space of discriminant
function that allows subsequent samples to be placed with confidence in one group or
another.

       Thus, the present invention provides a method for identifying protein profiles

10   which are specific for a disease or an individual. The diseases which can be diagnosed and
monitored using the novel method described herein include, but are not limited to,
schizophrenia, Alzheimer's disease, Parkinson's disease, multiple sclerosis, manic
depression, Huntington's disease, viral encephalitis, stroke, dementia, headaches, bacterial
meningitis, insomnia and transmissible spongiform encephalopathies.

15

A. Protein Profile for Alzheimer's Disease (AD)

       In one embodiment, a protein profile specific for AD is identified. At least about
18 protein spots have been found to make up a protein profile. These spots, AD-1 through
AD-18 are identified by their molecular weight and isoelectric point in Table 1. The AD

20   profile identified herein is comprised of 18 qualitative markers. Another profile specific
for AD has been identified using a combination of proteins that include 2 of those
qualitative markers with 7 other proteins that are altered in various diseases.

B. Protein Profile for Parkinson's Disease (PD)

       The present invention also includes a protein profile for PD. The present inventor

25   has discovered at least 3 qualitative and 5 quantitative proteins making up a disease-
specific protein profile. The 3 qualitative markers of the profile (i.e. spots that are not
present in normal controls) are protein 129, CIT-A (molecular weight of approximately 94
kDa and isoelectric point of approximately 5.2), and CIT-B (molecular weight of
approximately 54 kDa and isoelectric point of approximately 5.58). The quantitative

30   markers, or spots which increase or decrease compared to normal are: Apo-A1 lipoprotein;
alpha 1 microglobulin subunit; two PGDS isoforms and complement 4 gamma. All spots
decrease in PD patients except one PGDS isoform (CIT-2) which is increased.

C. Protein Profile for Schizophrenia

In another embodiment, a protein profile specific for schizophrenia is identified. As described in the Examples, using the methods of the present invention a schizophrenia-specific profile made up of seven proteins was identified. These seven proteins, protein 91, protein 48, protein 46, orosomucoid, prostaglandin D synthetase (PGDS), antithrombin III and fibrinogen degradation products are altered in schizophrenic subjects when compared to normal controls. In particular, protein 91, orosomucoid (also known as α_1-acid glycoprotein), PGDS, and antithrombin III have a relative concentration which is decreased relative to normal. Proteins 48 and 46 are increased relative to normal. Fibrinogen degradation products are absent in normal controls and appear in schizophrenic samples.

D. Differentiation between Neurologic Disorders

Once a protein profile has been identified, this group of proteins can be used to define the multidimensional space of discriminant function that allows subsequent samples to be placed with confidence in one group or another. As shown in Figures 5, 6 and 11, multiple discriminant function analyses can be used to distinguish between various neurological conditions. Figure 5 shows that PD patients (c) and AD patients (a) can be distinguished from each other and from healthy controls (b) where the multidimensional discriminating space is plotted in 2-dimensions. Similarly, in Figure 6, PD patients (c) separate from AD (b) and from controls (a). Figure 11 illustrates the diagnosis of subacute sclerosing panencephalitis (SSPE) patients in Group 5, individually labeled E with complete resolution from the other patient groups.

In other embodiments, protein profiles specific for multiple sclerosis, Parkinson's disease, subacute sclerosing panencephalitis (SSPE) or Creutzfeldt-Jakob disease are disclosed. The ability to distinguish between SSPE and multiple sclerosis is an especially interesting and novel finding as conventional biochemical techniques cannot distinguish between these two disorders.

II.   Diagnosis of Neurologic Disorders Using Disease-Specific Protein Profiles

The disease-specific protein profile obtained as described above can then be used
for diagnosis of the specific disorder. The individual proteins of the protein profiles can be
detected or quantified by any of a number of means well known to those of skill in the art.
These may include visualization techniques, analytic biochemical methods such as
electrophoresis, capillary electrophoresis, high performance liquid chromatography
(HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, partial amino
acid sequence determination and the like, or various immunological methods such as fluid
or gel precipitation reactions, immunodiffusion (single or double), immunoelectrophoresis,
radioimmunoassay (RIA), enzyme–linked immunosorbent assays (ELISAs),
immunofluorescent assays, western blotting, and the like.

In one aspect, a qualitative change in one or more proteins of the protein profile is
determined. Qualitative changes include the appearance of a protein spot that is not
detectable in samples obtained from normal controls or the disappearance of a protein spot
which is detectable in normal controls but not in the sample taken from an affected subject.

In another aspect, a quantitative change in one or more proteins of the profile is
measured. Preferably, the protein profile is quantified using 2DE, immunoassay or
Western blotting. The concentration of protein levels may be expressed in absolute terms,
for example, optical density as read by image analysis. Alternatively, the concentrations
can be expressed as a fraction, relative to normal levels of the same protein.

The proteins which make up the protein profile can be individually isolated and,
optionally purified. Methods of isolating and purifying proteins from 2DE gel are known
in the art. The isolated proteins are then identified, for example, by partial amino acid
sequence, mass spec or carbohydrate analysis. Monoclonal or polyclonal antibodies can
then be generated to the isolated proteins by methods known in the art. *See* Harlow and
Lane (1988) *Antibodies. A Laboratory Manual,* Cold Spring Harbor Publications, New
York, for a description of methods of generating antibodies. Used in combination, these
specific antibodies are used to diagnosis the specific disease in a subject by analyzing a
biological sample from the subject.

The collection of biological sample and subsequent testing for a disease-specific
protein profile is discussed in more detail below.

A)   Sample Collection and Processing

The disease-specific protein profile is preferably quantified in a biological sample derived from a mammal, more preferably from a human patient. A biological sample is a sample of biological tissue or fluid that contains a protein profile concentration that may be correlated with protein profile levels of a healthy control. Particularly preferred biological samples include but are not limited to, plasma, urine, serum, neurological tissue and cerebrospinal fluid.

The biological sample may be pretreated as necessary by dilution in an appropriate buffer solution or concentrated, if desired. Any of a number of standard aqueous buffer solutions, employing one of a variety of buffers, such as phosphate, Tris, or the like, at physiological pH can be used.

In a preferred embodiment, assays are performed using cerebrospinal fluid (CSF). Obtaining and storing CSF are well known to those of skill in the art. Typically CSF is obtained by lumbar puncture. The CSF may be diluted by the addition of buffers or other reagents well known to those of skill in the art and may be stored for up to 24 hours at 2–8°C, or at –20°C or lower for longer periods, prior to measurement of the protein profile. In a particularly preferred embodiment, the CSF is stored at –70°C without preservative indefinitely.

B)   Electrophoretic Assays

As indicated above, a protein profile specific for schizophrenia may be obtained form cerebrospinal fluid using electrophoretic methods. Means of detecting proteins using electrophoretic techniques are well known to those of skill in the art (see generally, R. Scopes (1982) *Protein Purification,* Springer–Verlag, N.Y.; Deutscher, (1990) *Methods in Enzymology Vol. 182: Guide to Protein Purification.,* Academic Press, Inc., N.Y.). In a preferred embodiment, the protein profile is obtained using two-dimensional electrophoresis. A particularly preferred separation relies on isoelectric focusing (IEF) in carrier ampholyte gradient gels or immobilized pH gradients for one dimension and 14% polyacrylamide gels for the second dimension. A detailed protocol for two-dimensional electrophoresis is provided in Examples below.

Proteins separated on 2DE gels can be visualized by any method known in the art. For example, Coomassie blue staining, gold staining, silver staining and the like may be

used to visualize proteins directly on the gels. (see, generally, *Current Protocols in Molecular Biology*, Ausubel *et al.* eds. (1996) § 10.6 to 10.8 and references therein). Alternatively, the proteins on the 2DE gel can be transferred or blotted onto a membrane and probed with an antibody. Methods of blotting and immunostaining are known to those of skill in the art. (see, e.g., Ausubel (1996), *supra*, § 10.8). In a preferred embodiment, the present invention obtains a protein profile by silver staining and, subsequently, by immunostaining.

C)   Immunological Binding Assays

In another embodiment, the protein profile is obtained or measured using any of a number of well recognized immunological binding assays *(see, e.g.,* U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, see also *Methods in Cell Biology Volume 37. Antibodies in Cell Biology*, Asai, ed. Academic Press, Inc. New York (1993); *Basic and Clinical Immunology* 7th Edition, Stites & Terr, eds. (1991).

Immunological binding assays (or immunoassays) typically utilize a "capture agent" to specifically bind to and often immobilize the analyte (in this case a protein of the specific protein profile). The capture agent is a moiety that specifically binds to the analyte. In a preferred embodiment, the capture agent is an antibody that specifically binds a protein of the protein profile.

Antibodies may be produced by any of a number of means well known to those of skill in the art (see, *e.g. Methods in Cell Biology Volume 37 Antibodies in Cell Biology*, Asai, ed. Academic Press, Inc. New York (1993); and *Basic and Clinical Immunology* 7th Edition, Stites & Terr, eds. (1991)). The antibody may be a whole antibody or an antibody fragment. It may be polyclonal or monoclonal, and it may be produced by challenging an organism (*e.g.* mouse, rat, rabbit. *etc.)* with a protein of the profile or an epitope derived therefrom. Alternatively, the antibody may be produced *de novo* using recombinant DNA methodology. The antibody can also be selected from a phage display library screened against the protein or interest (see, *e.g.* Vaughan *et al.* (1996) *Nature Biotechnology*, 14:309–314 and references therein).

Immunoassays also often utilize a labeling agent to specifically bind to and label the binding complex formed by the capture agent and the analyte, The labeling agent may

itself be one of the moieties comprising the antibody/analyte complex. Thus, the labeling agent may be a labeled profile protein or a labeled anti-profile protein antibody, Alternatively, the labeling agent may be a third moiety, such as another antibody, that specifically binds to the antibody/protein complex.

5   Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G may also be used as the label agent. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species. *See*, generally Kronval, *et al. J. Immunol.;* 111:1401–1406 (1973), and Akerstrom, *et al., J.*
10   *Immunol.,* 135:2589–2542 (1985).

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, analyte, volume of solution, concentrations, and the like.

15   Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

1.   Non–Competitive Assay Formats

Immunoassays for detecting proteins may be either competitive or noncompetitive.
20   Noncompetitive immunoassays are assays in which the amount of captured analyte (in this case the protein profile) is directly measured. In one preferred "sandwich" assay, for example, the capture agent can be bound directly to a solid substrate where they are immobilized. These immobilized antibodies then capture the protein of interest present in the test sample. The protein thus immobilized is then bound by a labeling agent, such as a
25   second antibody bearing a label. Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second can be modified with a detectable moiety, such as biotin, to which a third labeled, molecule can specifically bind, such as enzyme–labeled streptavidin.

30

2.   Competitive Assay Formats

In competitive assays, the amount of analyte (proteins of the protein profile) present in the sample is measured indirectly by measuring the amount of an added (exogenous) analyte displaced (or competed away) from a capture agent by the analyte present in the sample. In one competitive assay, a known amount of, in this case, the protein profile is added to the sample and the sample is then contacted with a capture agent. The amount of protein bound to the antibody is inversely proportional to the concentration of the protein present in the sample before the exogenous protein is added to the reaction mix.

In a particularly preferred embodiment, the antibody is immobilized on a solid substrate. The amount of protein bound to the antibody may be determined either by measuring the amount of protein present in an protein/antibody complex, or alternatively by measuring the amount of remaining uncompeted protein. The amount of protein may be detected by providing a labeled protein molecule.

A hapten inhibition assay is another preferred competitive assay. In this assay a known analyte, in this case the protein profile is immobilized on a solid substrate. A known amount of antibody is added to the sample, and the sample is then contacted with the immobilized protein. In this case, the amount of antibody bound to the immobilized protein is inversely proportional to the amount of protein present in the sample. Again the amount of immobilized antibody may be detected by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. Detection may be direct where the antibody is labeled or indirect by the subsequent addition of a labeled moiety that specifically binds to the antibody as described above.

3.   Other Assay Formats

In a preferred embodiment, Western blot (immunoblot) analysis is used to detect and quantify the presence of protein in the sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind protein. The anti-protein antibodies specifically bind to protein on the solid support. These antibodies may be directly labeled or alternatively may

be subsequently detected using labeled antibodies (*e.g.*, labeled sheep anti–mouse antibodies) that specifically bind to the anti-protein. A particularly preferred protocol for Western Blot detection of human or animal protein in CSF is provided in Example 1.

Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (*e.g.*, antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques *(see, Monroe et at.* (1986) *Amer. Clin. Prod. Rev.* 5:34–41).

4.   Labels

The particular label or detectable group used in the assay is not a critical aspect of the invention, so long as it does not significantly interfere with the specific binding of the antibody used in the assay. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well developed in the field of immunoassays and, in general, most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.* Dynabeads™), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, texas red, rhodamine, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.)* beads.

The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

Non–radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to the molecule. The ligand then binds to an anti–ligand (*e.g.*, streptavidin) molecule which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti–ligands can be used. Where

a ligand has a natural anti–ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti–ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

The molecules can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3–dihydrophtlialazinediones, e.g., luminol. (For a review of various labeling or signal producing systems which may be used, see, U.S. Patent No. 4,391,904).

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorophore with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen–coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

D) Diagnostic Imaging

One or more of the proteins making up the disease protein profiles obtained using the methods described herein can also be used as markers in diagnostic imaging techniques, for example magnetic resonance imaging (MRI).

III.   Determining Disease Severity Using the Protein Profile

One or more the proteins which make up the protein profiles as described herein
can also be useful in determining disease severity. In particular, an indicator of disease
severity can be the absolute or relative amount by which one or more proteins of the profile
have increased or decreased as compared to healthy control levels. For example, as shown
in Figure 4, panels A through C, severity of PD is correlated to decrease in CIT-5 (Apo-A1
lipoprotein). Panel A shows that the relative quantity of Apo-A1 lipoprotein decrease as
the "H+Y" rating of disease severity increases. The least severely affected patients (shown
as "1") have more Apo-A1 lipoprotein than the more severely affected patients (shown as
"2.5" or "3"). Panel B shows that a decrease in Apo-A1 lipoprotein also correlates to age
and duration of PD. Young patients with short disease duration ("YS") have relatively
more Apo-A1 lipoprotein than young patients with long duration ("YL") or old patients
with either short ("OS") or long ("OL") duration disease. Panel C expresses Apo-A1
lipoprotein amounts plotted against clinical parameters. A rating of "1" indicates short
duration PD with no dyskinesia, and no or minimum dopamine therapy. A "2" indicates
medium duration disease, dyskinesia and low dopaminergic therapy. A "3" indicates long
duration, dyskinesia and moderate to high dopaminergic therapy. As the rating increases,
the relative amount of Apo-A1 lipoprotein decreases.

IV.   Therapeutic Monitoring and Patient Management using Disease-Specific
Protein Profiles

The disease specific protein profiles which are identified as described herein are
also useful in therapeutic monitoring and patient management. For example, using the
schizophrenia protein profile described herein, changes in the levels of the individual
proteins in response to drug treatment can be evaluated. As shown in Figures 7 to 10,
when the protein profile of untreated schizophrenics is compared to those on some
conventional treatment regimes, differences in the levels of the individual proteins is
observed. In some cases, the treatments drive the protein level closer to normal (Figures 8
and 10). In this way, it is contemplated that new treatment regimes could be developed by
examining the levels of the protein profile. Similarly, the protein profiles of individual
subjects could be monitored for example, to determine progression of the disease. The

present invention, therefore, provide a rapid and efficient way to monitor treatment

regimes and to determine if an individual is deteriorating or improving.

The protein profiles are also useful in developing drugs using animal models of

disease. Human disease models can be created in laboratory animals, for instance, by

5   inserting a human gene(s) into the animal. The protein profiles obtained by the methods

described herein are applicable to these animal models and can be used to monitor

treatment protocols and regimes in these animal models.

The following examples are provided to illustrate but not limit the present

10   invention.

EXAMPLES

Example 1:   Preparation of Samples

Cerebrospinal fluid samples were kindly provided by various neurologists and

15   veterinarians. The diagnoses were made by the referring physicians according to standard

clinical criteria in addition to pathological studies as appropriate. The CSF was collected

and the samples were immediately frozen at -70°C before shipment to our laboratory.

Upon receipt, samples were thawed and aliquoted for use. Samples for 2-DE were

prepared by the addition of 9M urea, 2% 2-mercaptoethanol, 2% NP-40, 0.8% BioLyte pH

20   3-10 carrier ampholytes, and 0.002% Bromophenol blue. For SDS-PAGE, 50 μL CSF was

added to 20 μL Laemmli sample buffer, heated for 5 minutes at 95°C, and loaded.

Two-Dimensional Electrophoresis (2DE)

Samples were separated by two-dimensional electrophoresis and silver stained as

25   previously described Harrington *et al.* (1991) *Methods: A Companion to Methods in

Enzymology* 3:135-141. Two dimensional electrophoresis consisted of isoelectric focusing

followed by electrophoresis using a polyacrylamide gel. Isoelectric focusing was done in

immobilized pH gradients. The 180 mM linear pH 3-10 gradient (Pharmacia, LKB,

Piscataway, New Jersey, USA, catalog #80-1128-30) were used. These gradients were

30   rehydrated, electrophoresed, and equilibrated as described in Bjellqvist *et al.* (1993)

Electrophoresis 14:1375-1378 and Bjellqvist *et al.* (1993) *Electrophoresis* 14:1357-1365.

The second dimension was performed in 14% T polyacrylamide gels and silver stained as

described in Harrington *et al.* (1991) *Methods: A Companion to Methods in Enzymology*
3:98-108 or electroblotted as described in Towbin *et al.* (1979) *Proc. Nat'l. Acad. Sci.*
76:4350-4354 onto nitrocellulose (Schleicher & Schuell, catalog # 0830N) or PVDF
(Millipore, Massachusetts, USA). The blots were stained with Coomassie Brilliant Blue
5   R-250 or silver stained.

Evaluation of two dimensional gels

        Two-dimensional gel electrophoresis gels and blots were analyzed either by direct
visual comparison, by comigration studies, and/or with the aid of computer image analysis
10   software. GALtool (Solomon *et al.* (1993) *CABIOS* 9:133-139") or Melanie (BioRad).
The raw data file for each sample is then processed by computer-assisted algorithms. The
algorithm was performed as follows. First, the protein spots were identified using an edge
detector or peak identifier. The x and y co-ordinates corresponding to pH and molecular
weight along with total integrated density were recorded. Next, all spots were compared
15   from one sample to another. A master gel was used to retain all the data.

        The compared gel data was then split into groups of each disease and normal
controls. Similarities and differences were noted. All of the different proteins were then
further analyzed in subsets of patients within each disease compared to controls.

        The above-described steps were subjected to multi-dimensional discriminant
20   function analysis using either BMDP™ 7M Mainframe programs (BMDP Statistical
Solutions Ltd., Cork, Ireland) or Unistat programs (Unistat House, London, England).
Linear and nonlinear discriminant function analyses were performed. All variable proteins
were examined for combinations that would provide the best discrimination and then the
best discrimination were investigated. Once these were determined, these proteins were
25   used to test a second, new population of samples. Only when these same proteins gave
discriminating power in the second population was this group of proteins considered for
the profile.

        Proteins altered in neurologic disorders that may not uniquely discriminate the
disease by themselves are studied to identify whether in combination a number of proteins
30   can be form a profile which is specific for neurologic disorders as described in Example 2.
The proteins can be identified by their migration in the gel. In addition, each protein in the
profile can be identified by obtaining a molecular footprinting, for example, by one or

more of the following: antibody assay, protein sequence determination, mass spectrometry
and database comparison.

Example 2:   Preparation of Specific Protein Profiles

5   **1. Alzheimer's Disease (AD)**

Six samples from AD patients were used in the computation described in Example
1. The profile was composed of 18 proteins, or spots, designated AD-1 through AD-18
which were altered in presence or amount from four healthy controls. All 18 spots present
in the AD samples were not present in healthy controls. AD-1 through AD-18 are

10   identified by name or by approximate molecular weight and isoelectric point in Table 1.
Another profile specific for AD has been identified using a combination of proteins that
include 2 of those qualitative markers with 7 other proteins that are altered in various
diseases.

          2. Parkinson's Disease (PD)

15   Six samples from PD patients were used to determine a protein profile as described
above. Eight proteins were found to be qualitatively or quantitatively altered in PD
samples when compared to normal controls. The protein spots are identified in Table 1.
CIT-A, CIT-B and CIT-C were present in PD samples and were not present in healthy
controls. CIT-1 (alpha 1 microglobulin), CIT-3 (PGDS isoform), CIT-4 (complement 4

20   gamma) and CIT-5 (Apo-A1 lipoprotein) were decreased in PD samples as compared to
controls, while CIT-2 (PGDS isoform) was increased in PD samples.

          3. Schizophrenia

Twenty samples from schizophrenic patients were used in the computation

25   described in Example 1. The profile was composed of seven proteins, or spots, which were
altered in presence or amount from 100 healthy controls matched for age and sex and 70
disease controls having disorders including manic depression, CJD, viral encephalitis,
subacute sclerosing panencephalitis, Korsakoff's psychosis, benign essential tremor,
Huntington's disease, cerebrovascular disease, Alzheimer's disease, motor neuron disease,

30   bacterial meningitis, systemic lupus erythematosis and headache. Results are shown in
Tables 2 and 3.

Table 2: Schizophrenia

Protein identification	Concentration (relative to normal levels)
protein 91	0.54
protein 48	1.27
orosomucoid	0.63
prostaglandin D synthetase (PGDS)	0.8
antithrombin III	0.7
protein 46	1.23

Table 3

Protein identification	Qualitative Changes (in percentage of patient population)
fibrinogen degradation products	30%

As these results indicate, a protein profile specific for schizophrenia is characterized by decreased amounts of PGDS, orosomucoid, antithrombin III and protein 91 as well as increased amounts of protein 48 and protein 46 when compared to normal CSF levels. Fibrinogen degradation products are absent in normal controls and appear in samples obtained from schizophrenics.

4.   **Multiple Sclerosis**

Twenty samples from multiple sclerosis patients were used in the computation described in Example 1. One hundred age and sex matched controls were used along with 70 other disorders including manic depression, CJD, viral encephalitis, subacute sclerosing panencephalitis, Korsakoff's psychosis, benign essential tremor, Huntington's disease, cerebrovascular disease, Alzheimer's disease, motor neuron disease, bacterial meningitis, systemic lupus erythematosis and headache. Using the methods described above, a protein profile for multiple sclerosis was obtained and is shown in Table 4 and 5.

Table 4: Multiple Sclerosis

Protein identification	Concentration (relative to normal levels)
ApoD (protein 108)	1.56
ApoD (protein 110)	2
proteins 91 and 92	2
α_1 β-glycoprotein	2

Apo J/K	1.6
transthyretin	1.4
C4γ	2.5
Ig K and L chains	2

Table 5: Multiple Sclerosis

Protein identification	Qualitative Changes (in percentage of patient population)
fibrinogen degradation products	13%

The protein profile for multiple sclerosis includes ApoD (protein 108), ApoD (protein 110), protein 91, protein 92, α_1 β-glycoprotein, ApoJ/K, transthyretin, C4γ, and Ig K and L chains. In affected individuals levels of these proteins are elevated as compared to normal controls. Fibrinogen degradation products also appear in these subjects.

5.   **Creutzfeldt-Jakob Disease (CJD)**

Twenty samples of CJD were used to compute a protein profile. The samples were matched by age and sex to 100 normal controls and to 70 disease controls having disorders including manic depression, multiple sclerosis, viral encephalitis, subacute sclerosing panencephalitis, Korsakoff's psychosis, benign essential tremor, Huntington's disease, cerebrovascular disease, Alzheimer's disease, motor neuron disease, bacterial meningitis, systemic lupus erythematosis and headache. As previously discussed, 99% of CJD patients show the appearance of 14-3-3 protein when compared to normal controls. In addition, 67% show the appearance of fibrinogen degradation products.

6.   **Subacute Sclerosing Panencephalitis (SSPE)**

Using 8 samples in the methods described above, a protein profile composed of proteins APOA1 isoforms, APOJ isoforms, isoforms of haptoglobin β-chain, 46, GC globulin, 91, C3-activator and Ig kappa and lambda chains was determined for SSPE. Figure 5 where the multidimensional discriminating space is plotted in 2-dimensions and illustrates the diagnosis of subacute sclerosing panencephalitis (SSPE) patients in Group 5, individually labeled E with complete resolution from the other patient groups. These results indicate that the present method can distinguish between SSPE and multiple sclerosis, two distinct disorders which cannot be differentiated using standard biochemical analyses.

Example 3:   Response of Schizophrenia Protein Profile Markers to Drug Treatment

       The seven protein markers which make up the protein profile identified in Example

2 were analyzed to determine if their levels were affected by drug treatment. Protein

quantity of each marker from normal controls (normal), untreated schizophrenics (none),

and schizophrenics taking fluphenazine and chloropromazine (Flu + CPZ) or fluphenazine

and lithium (Flu + Li) or fluphenazine alone (Flu). As shown in Figures 2 and 4, levels of

protein 48 and protein 46 in schizophrenics taking drugs is closer to normal levels than in

untreated patients. Interestingly, as shown in Figure 1, protein 91, which is decreased in

the CSF of schizophrenia patients, is further decreased in schizophrenic patients who are

following drug treatment.

       PGDS levels are decreased in both treated and untreated schizophrenic patients,

however, in patients taking fluphenazine alone or fluphenazine and lithium, the levels of

PGDS are closer to normal controls. (Figure 3). The inventor has previously shown that

altered levels of PGDS glycoforms are correlated with symptoms of sleep disorders. The

present invention also indicates how PGDS levels correlate with sleep disorders. The

schizophrenic patients who were on treatment regimes in which PGDS levels were closer

to normal also had fewer sleep related symptoms. Thus, the present invention provides a

method of monitoring treatment regimes and correlating protein levels to symptoms.

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CLAIMS

1.   A method of diagnosing a neurological disorder in a biological sample, the method comprising:

(a) obtaining a disease-specific protein profile by detecting proteins whose levels are altered in a subject having a neurological disorder when compared to levels of a healthy control;

(b) obtaining the biological sample;

(c) measuring the levels of the disease-specific protein profile obtained in step (a) in the biological sample; and

(d) comparing the levels of the disease-specific protein profile in the biological sample to levels found in the healthy control;

wherein altered levels of the protein profile of the biological sample as compared to the healthy control is diagnostic of the neurological disorder.

2.   The method according to claim 1, wherein the biological sample is cerebrospinal fluid.

3.   The method according to claim 1, wherein the neurologic disorder is selected from the group consisting of schizophrenia, Parkinson's disease (PD) and Alzheimer's Disease (AD).

4.   The method according to claim 1, wherein the disease-specific protein profile is obtained using 2 dimensional gel electrophoresis (2DE).

5.   The method according to claim 1, wherein the disease-specific protein profile is obtained by Western blotting.

6.   The method according to claim 1, wherein the disease-specific protein profile is obtained by immunoassay.

7.   The method according to claim 1, wherein in step (c) the protein profile is measured by visualizing a 2DE gel.

8. The method according to claim 1, wherein in step (c) the protein profile is measured using immunoassay.

5   9. The method according to claim 1, wherein in step (c) the protein profile is measured using Western blotting.

10. The method according to claim 1 wherein the neurologic disorder is Alzheimer's Disease (AD).

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11. The method according to claim 10 wherein the protein profile comprises proteins AD1 through AD18.

12. The method according to claim 11, wherein proteins AD1 through AD18

15   are present or increased in samples obtained from AD patients as compared to samples obtained from healthy controls.

13. The method according to claim 1 wherein the neurologic disorder is Parkinson's disease.

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14. The method according to claim 13 wherein the protein profile comprises CIT-A, CIT-B, CIT-C (protein 129), CIT-1 (alpha 1 microglobulin subunit), CIT-2 (PGDS isoform), CIT-3 (PGDS isoform), CIT-4 (complement 4 gamma) and CIT-5 (Apo-A1 lipoprotein).

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15. The method according to claim 14 wherein CIT-A, CIT-B and CIT-C are present in PD patients and absent in healthy controls, CIT-2 is increased in PD patients as compared to healthy controls and CIT-1, CIT-3, CIT-4 and CIT-5 are decreased in PD patients as compared to healthy controls.

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16. The method according to claim 1 wherein the neurologic disorder is multiple sclerosis.

17.   The method according to claim 15 wherein the protein profile comprises ApoD (protein 108), ApoD (protein 110), protein 91, protein 92, α_1 β-glycoprotein, ApoJ/K, transthyretin, C4γ, and Ig K and L chains.
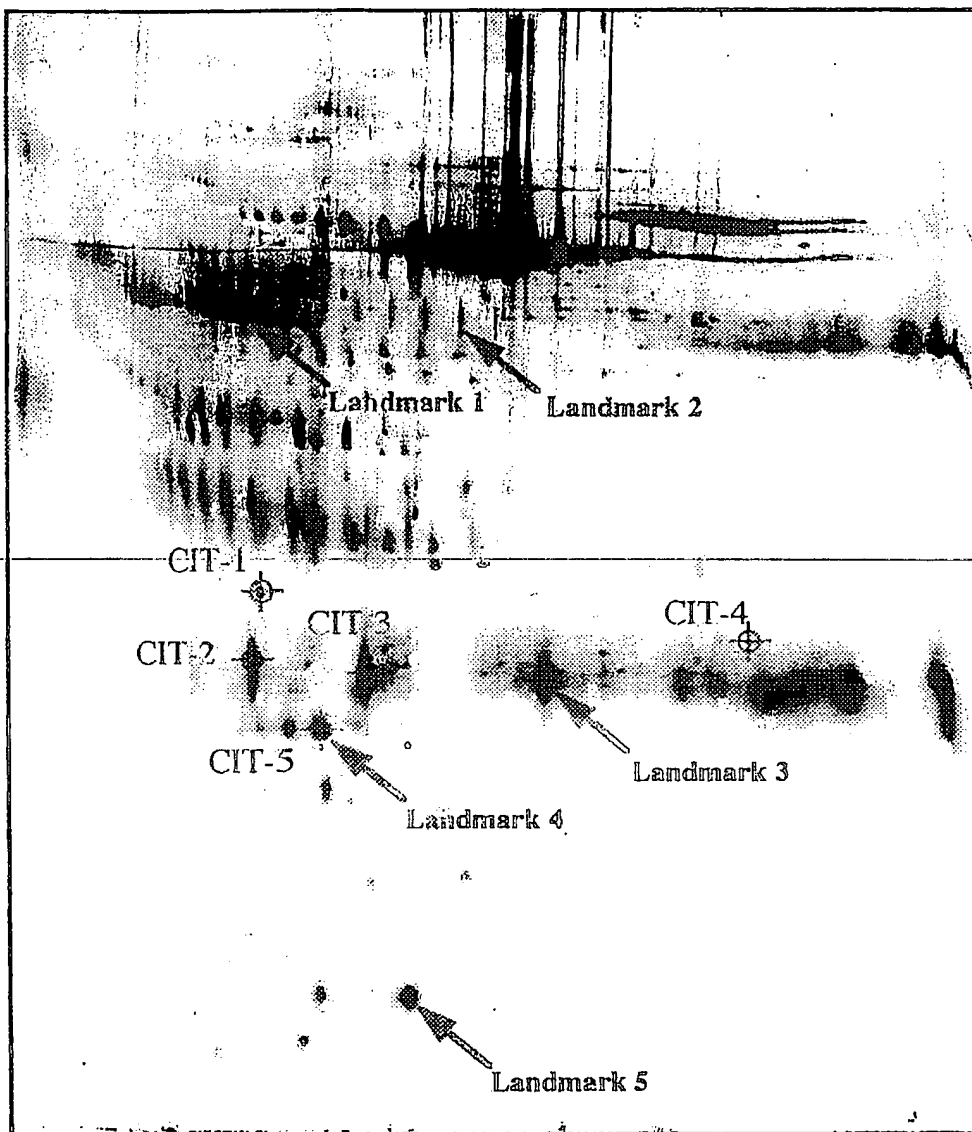
18.   The method according to claim 1 wherein the neurologic disorder is subacute sclerosing panencephalitis (SSPE).

19.   The method according to claim 17 wherein the protein profile comprises APOA1 isoforms, APOJ isoforms, isoforms of haptoglobin β-chain, 46, GC globulin, 91, C3-activator and Ig kappa and lambda chains.
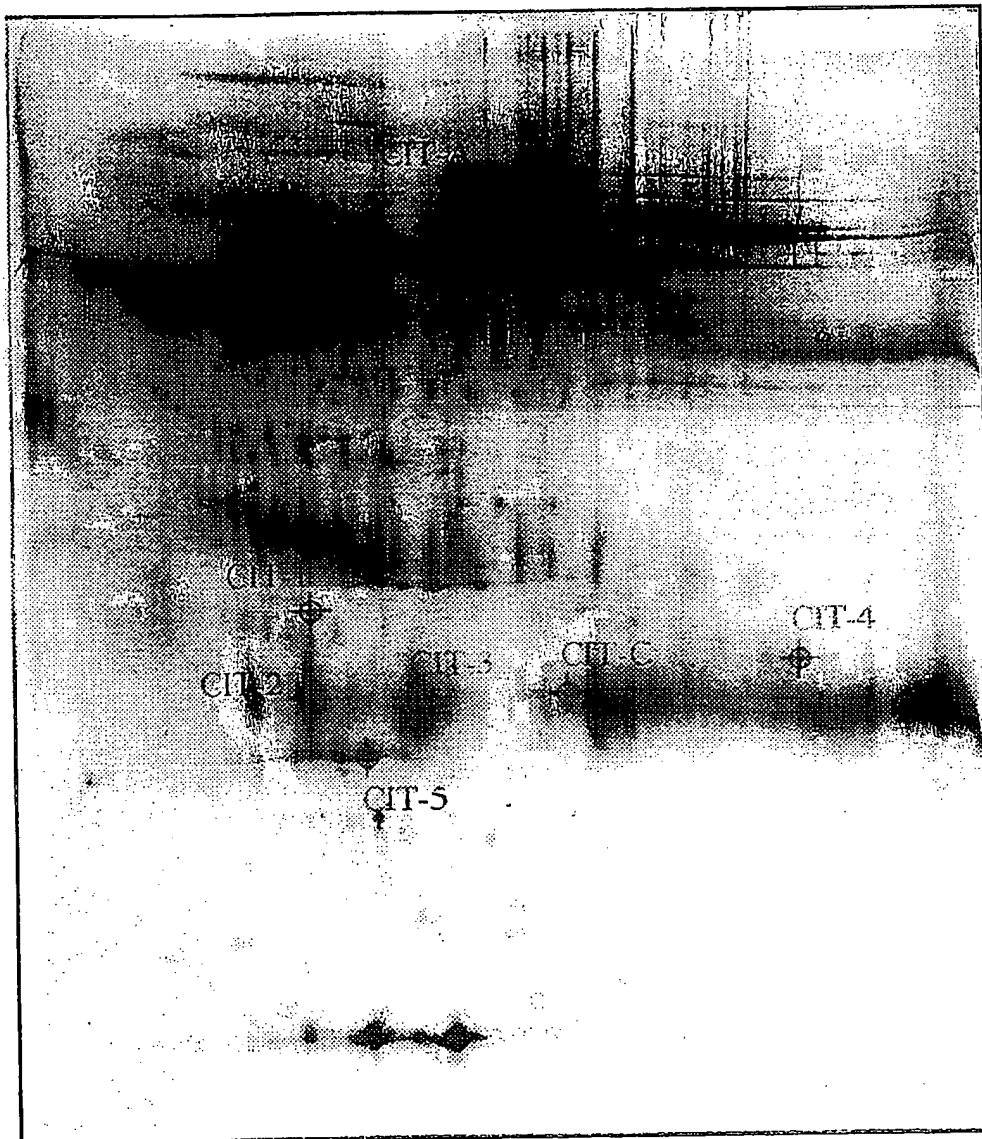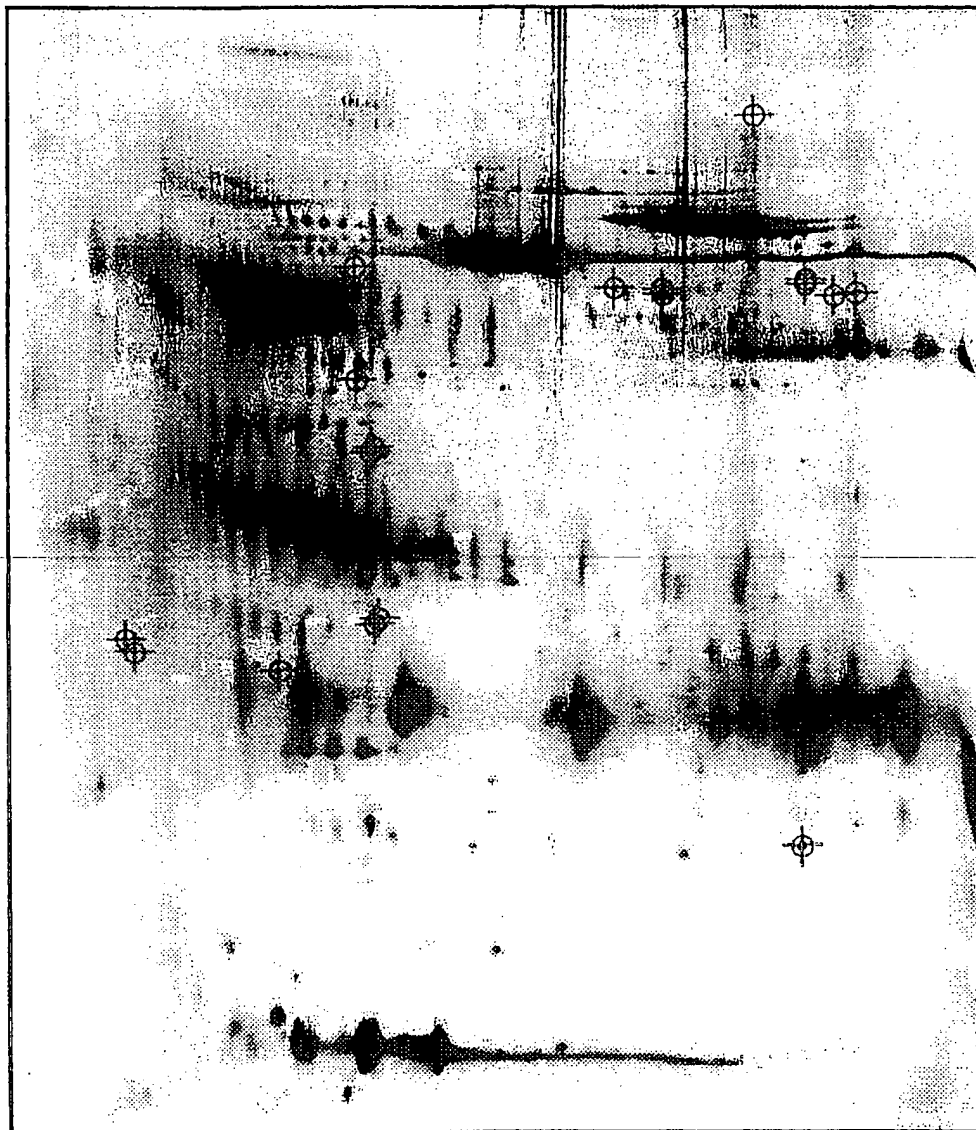
Normal

FIG 1

Parkinson's Disease

FIG 2
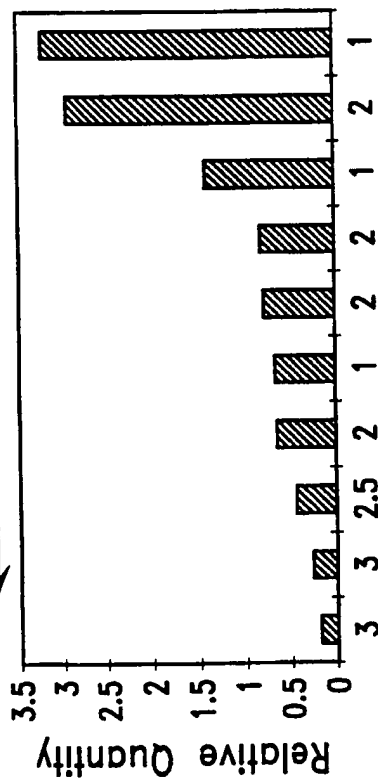
Alzheimer's Disease

FIG 3

Fig. 4B

CIT-5 vs Onset Age/Duration

Relative Quantity

x-axis labels: OL OL OL YL OS OL YL YS YS YS YS

y-axis: 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5

Fig. 4A

CIT-5 vs H+Y Rating

Relative Quantity

x-axis labels: 3 3 2.5 2 1 2 2 1 2 1

y-axis: 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5
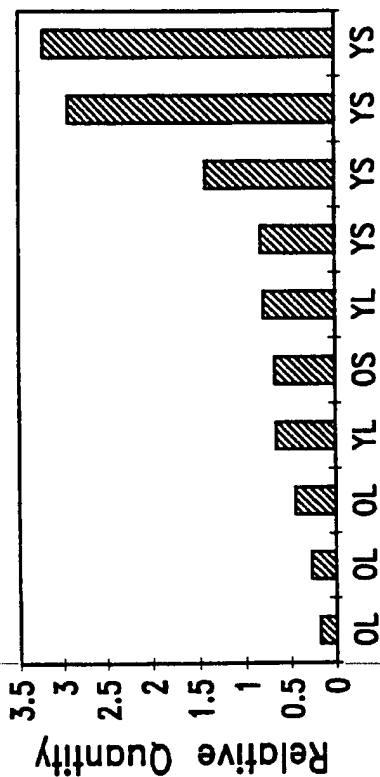
Fig. 4C

CIT-5 vs Clinical Parameters

Relative Quantity

x-axis labels: 3 3 2 3 3 1 1 1 1 1

y-axis: 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5
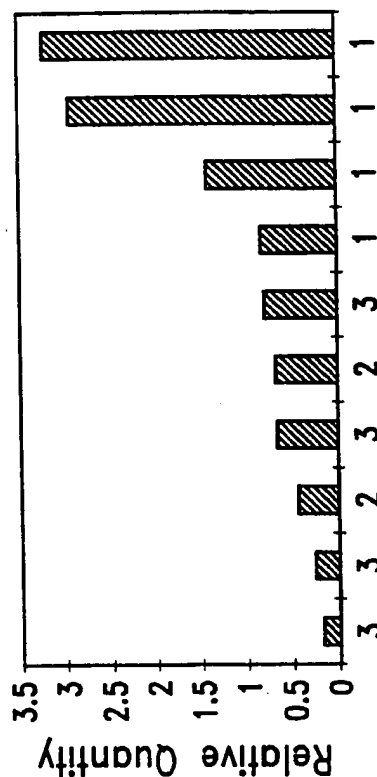
1 – Short duration, no dyskinesia, no/minimum dopaminergic therapy

2 – Medium duration, dyskinesia, low dopaminergic therapy

3 – Long duration, dyskinesia, high/moderate dopaminergic therapy
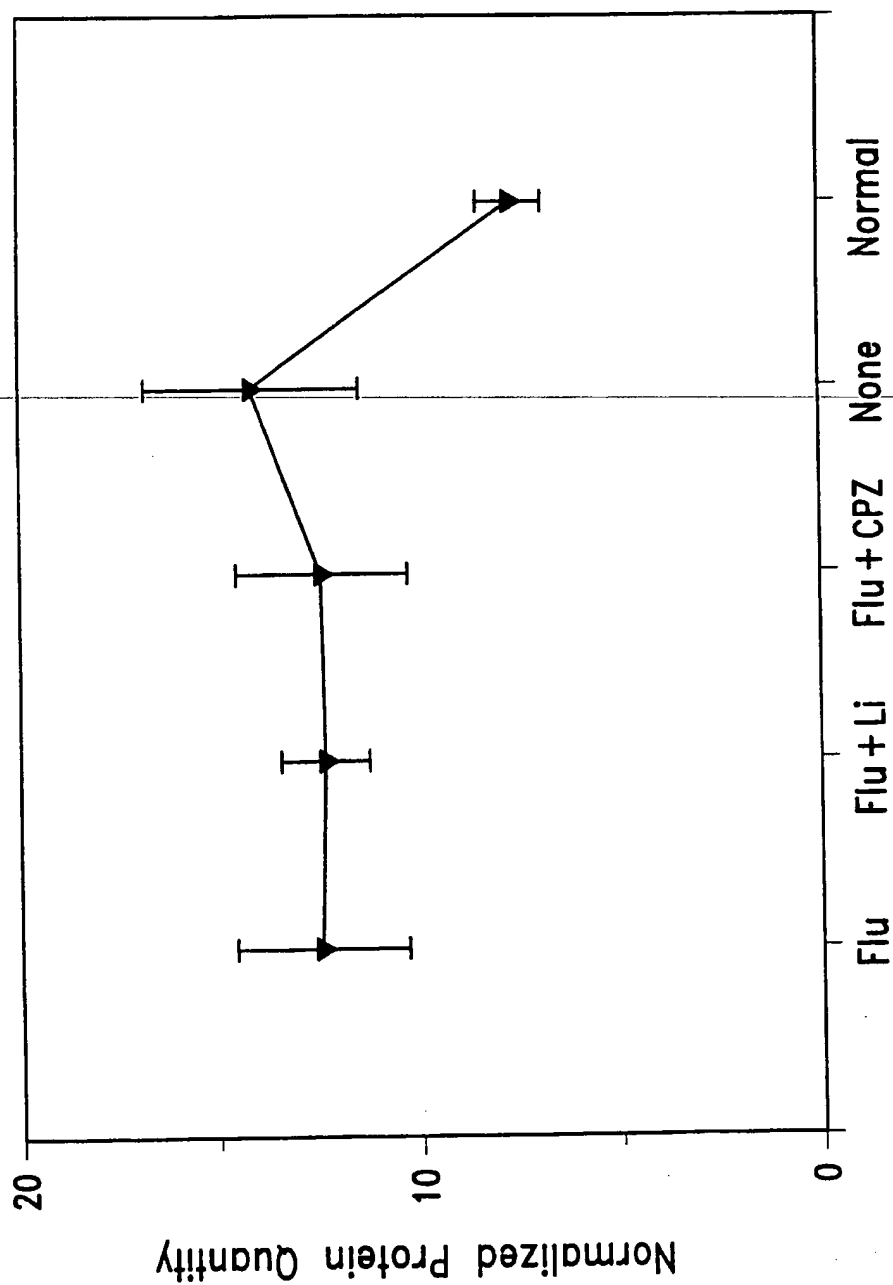
Fig. 5
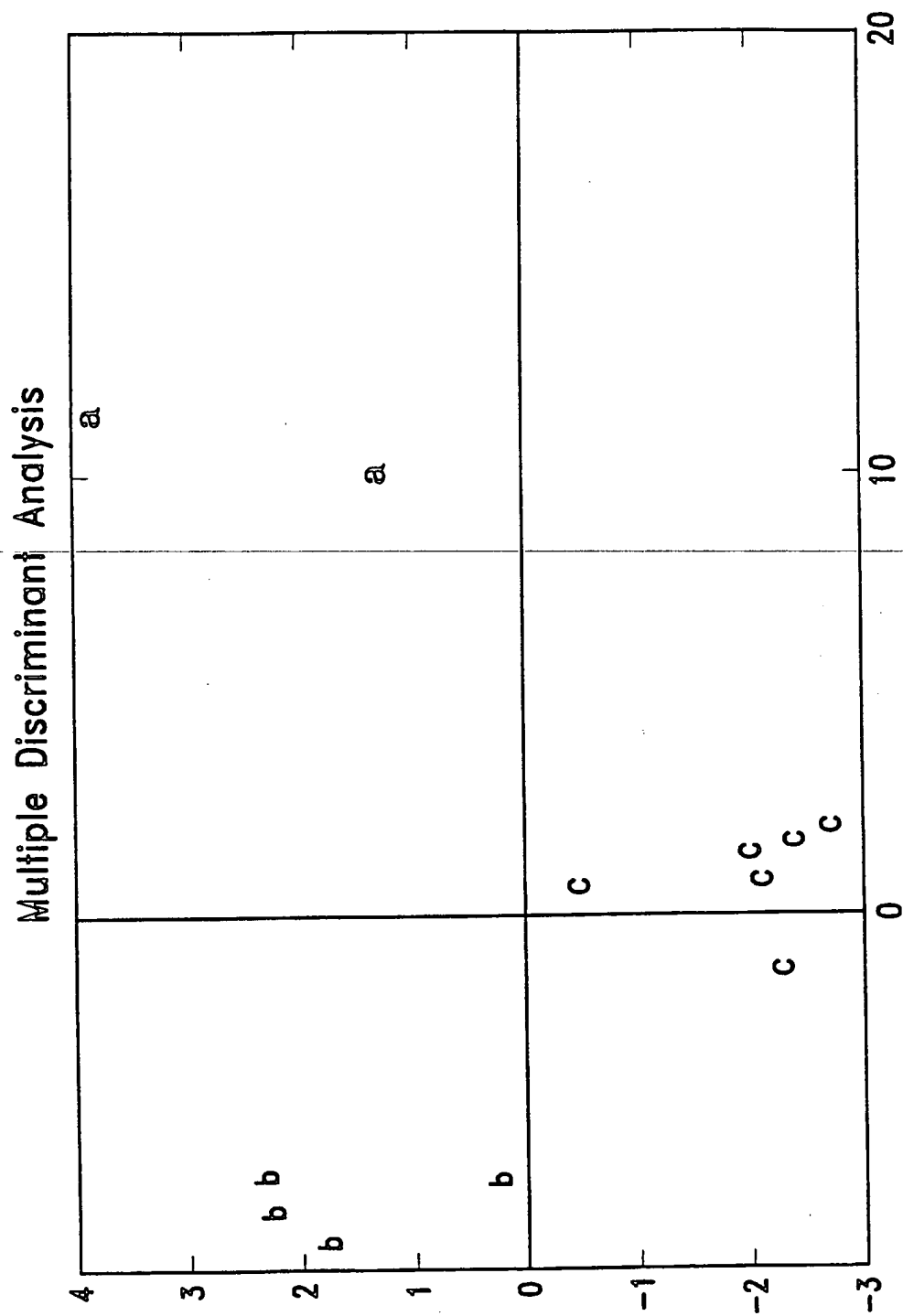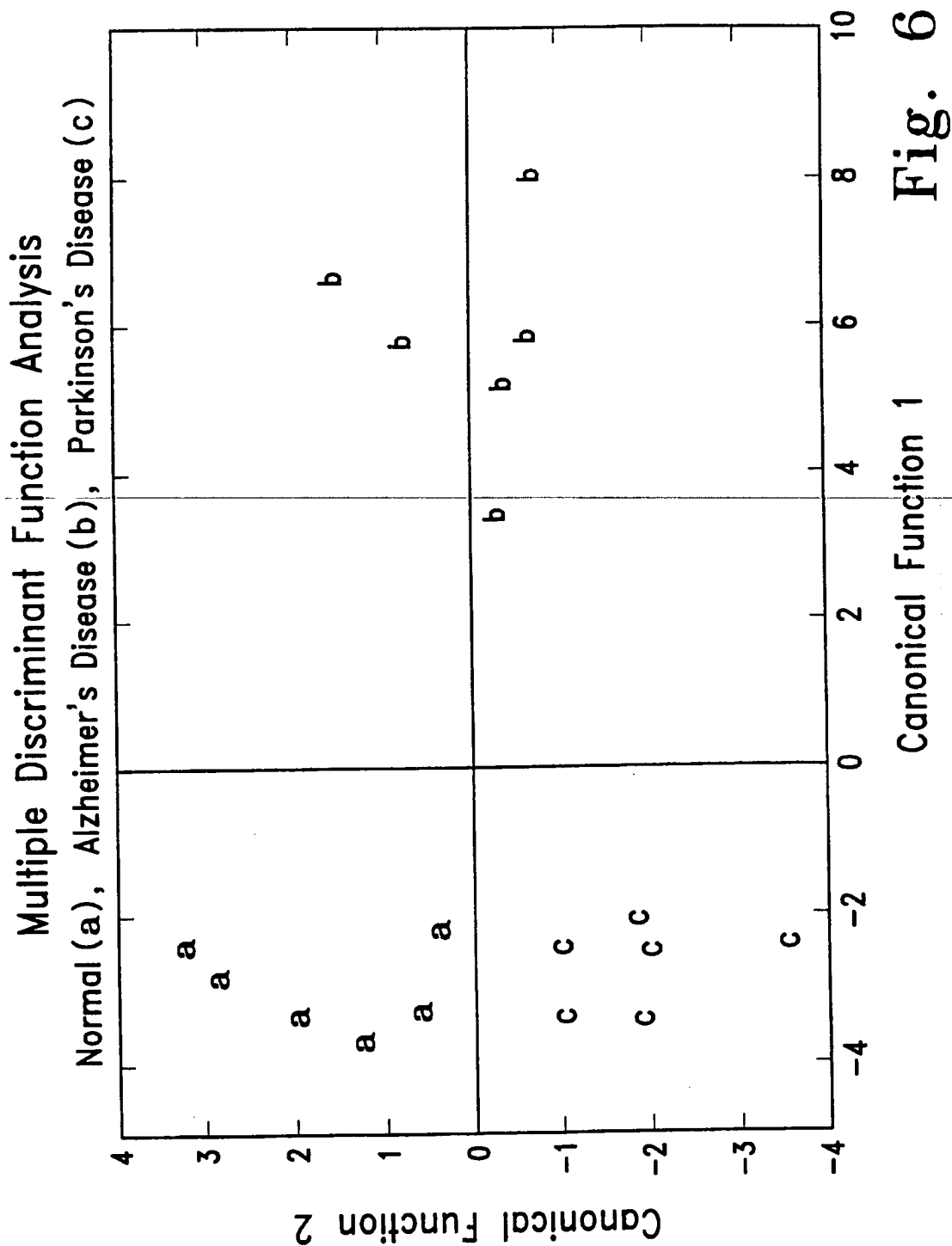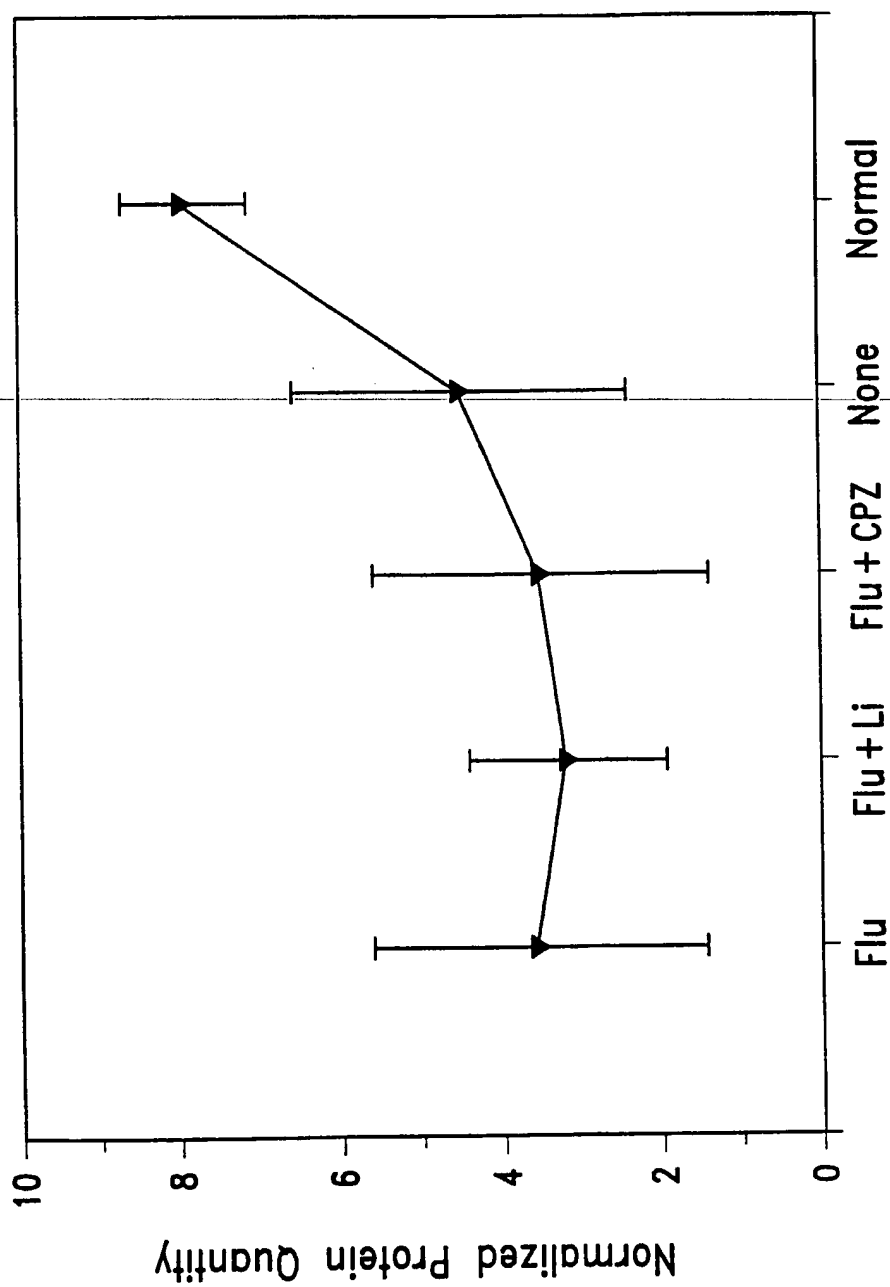
Fig. 6

Fig. 7

Fig. 8

Fig. 9

Fig. 10

FIG. 11

CANONICAL VARIABLE 2
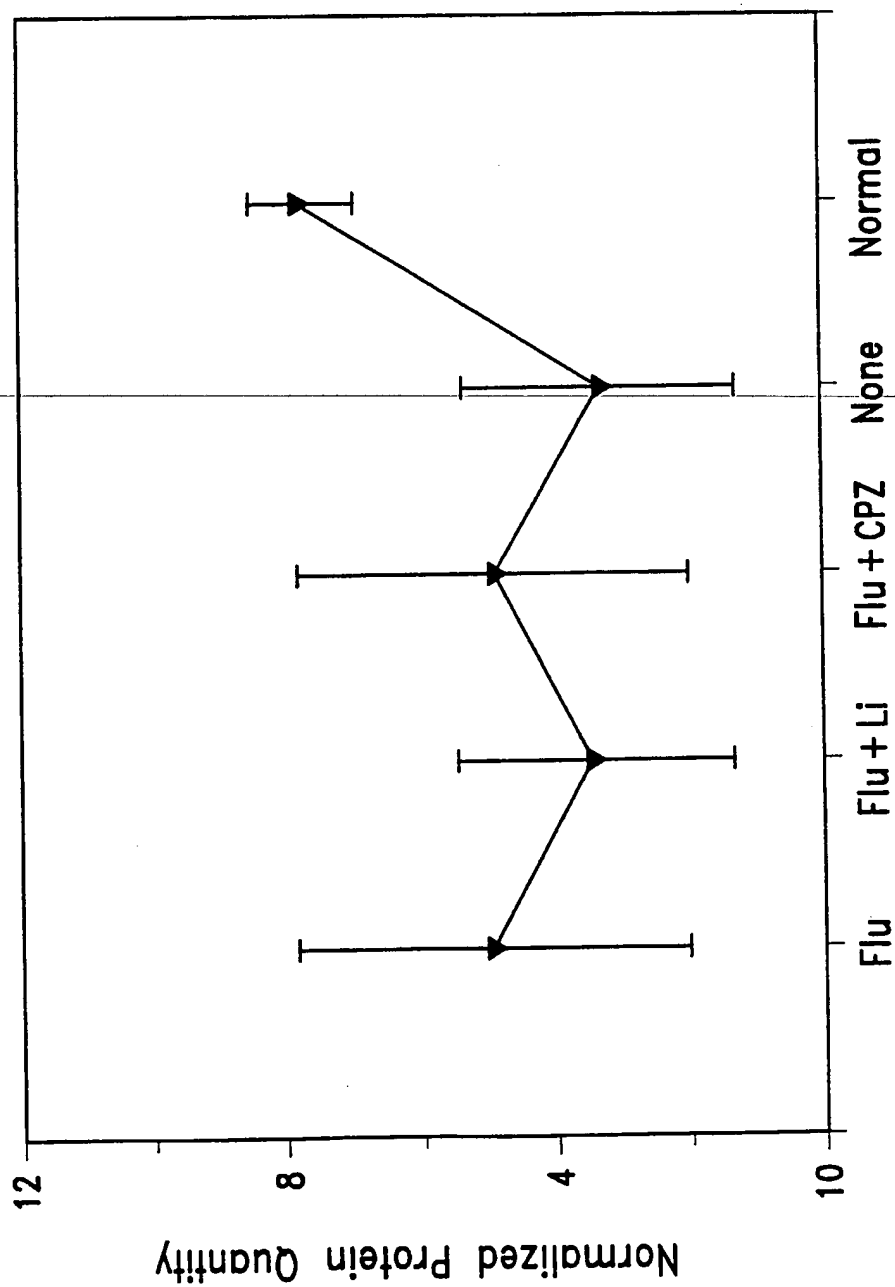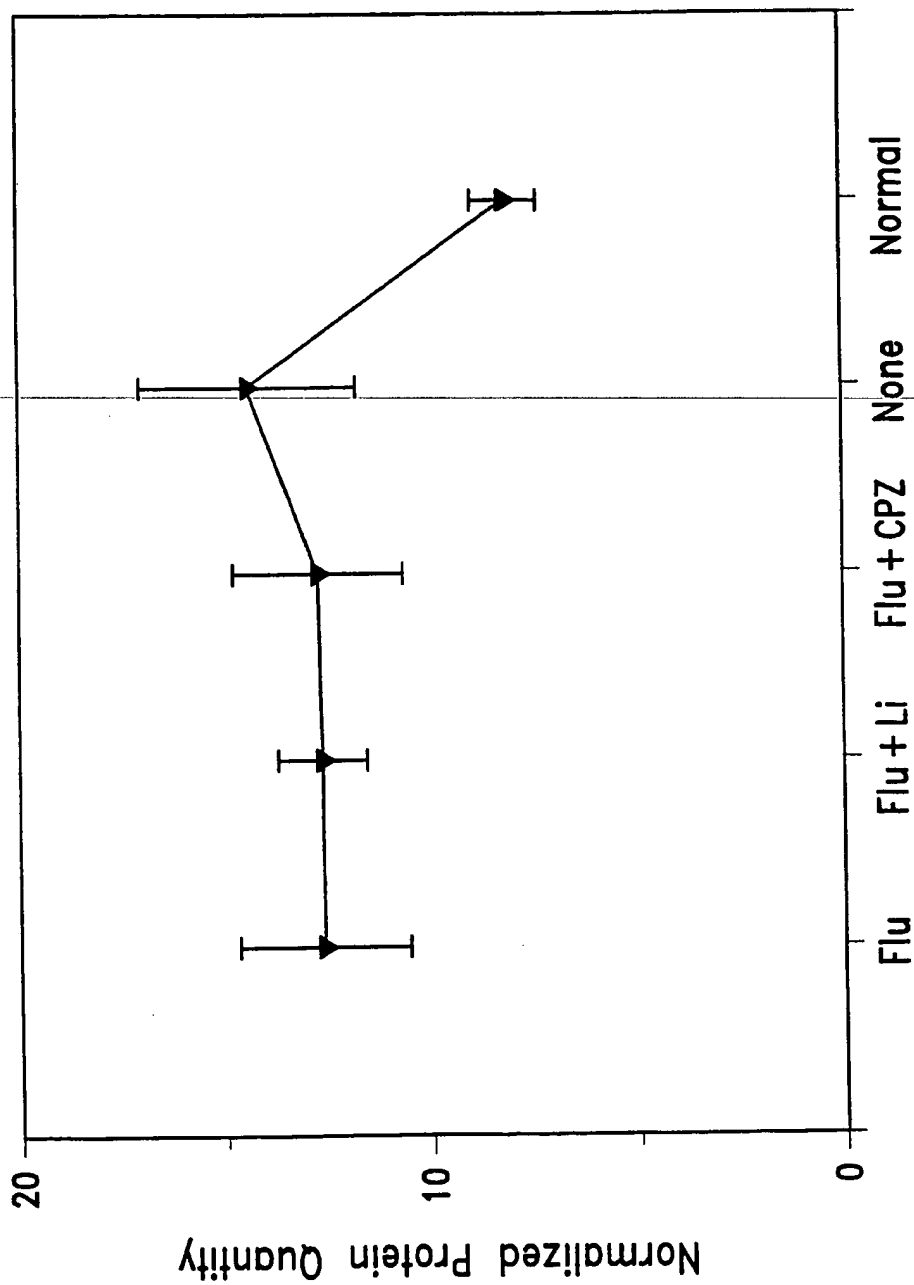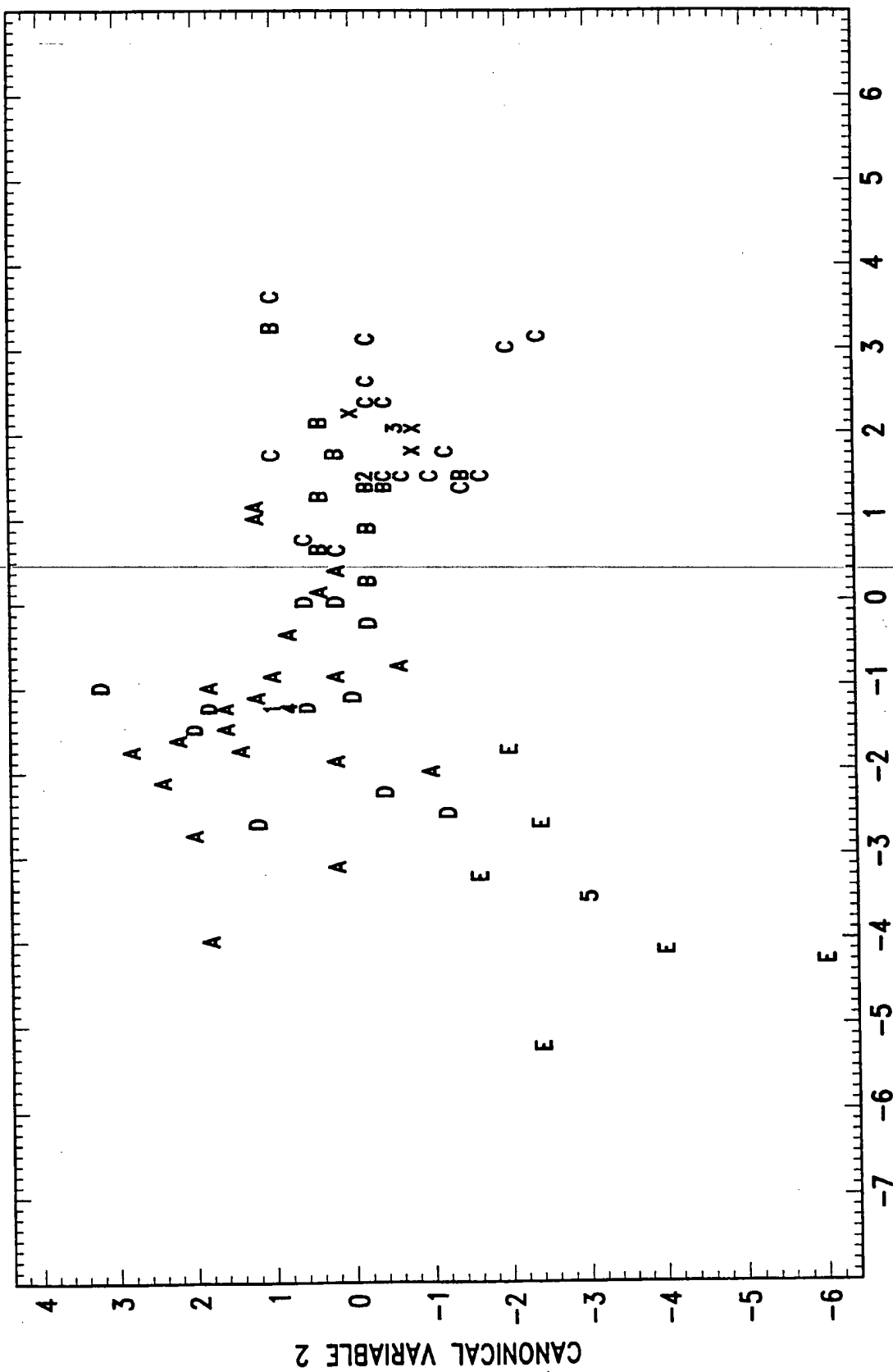
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   G01N33/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 33992 A (UNIV BAR ILAN ;MOR RESEARCH APPLIC LTD (IL); SHALIT FRANCES (IL)) 14 December 1995 see claims see page 4, line 9 - page 5, line 2 see page 6, line 23 - page 8, line 4 see page 9, line 19 - line 25 ---	1-19
X	WO 95 05604 A (MOLECULAR GERIATRICS CORP ;US GOVERNMENT (US)) 23 February 1995 cited in the application see claims 1,2,8,9 see page 3, line 6-10 see page 7, line 1 - line 13 see page 12, line 26 - page 13, line 23 --- -/--	1-19

X	Further documents are listed in the continuation of box C.		X	Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
7 July 1998	14/08/1998

Name and mailing address of the ISA	Authorized officer
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page 1 of 2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 13273 A (ESA INC) 6 August 1992 see claims 1-8 see page 8, line 1 - line 8	1-19
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X	WO 86 03006 A (ESA INC) 22 May 1986 see claims 28-31 see page 15, line 6 - page 16, line 3	1-19
X	BEHAN ET AL: "Serum Proteins, amyloid and Alzheimer's disease" J.AMERICAN GERIATRICS SOC., vol. 18, no. 10, - October 1970 pages 729-797, XP002070711 see table 1 see page 796	1-19

1

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Information on patent family members

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